Astrocyte elevated gene-1 (*AEG-1*) is a target gene of oncogenic Ha-ras requiring phosphatidylinositol 3-kinase and c-Myc

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It is well established that Ha-*ras* and c-*myc* genes collaborate in promoting transformation, tumor progression, and metastasis. However, the precise mechanism underlying this cooperation remains unclear. In the present study, we document that astrocyte elevated gene-1 (*AEG-1*) is a downstream target molecule of Ha-*ras* and c-*myc*, mediating their tumor-promoting effects. AEG-1 expression is elevated in diverse neoplastic states, it cooperates with Ha-ras to promote transformation, and its overexpression augments invasion of transformed cells, demonstrating its functional involvement in Ha-ras-mediated tumorigenesis. We now document that AEG-1 expression is markedly induced by oncogenic Ha-ras, activating the phosphatidylinositol 3-kinase signaling pathway that augments binding of c-Myc to key E-box elements in the *AEG-1* promoter, thereby regulating *AEG-1* transcription. In addition, Ha-ras-mediated colony formation is inhibited by *AEG-1* siRNA. This is a demonstration that Ha-ras activation of a tumor-promoting gene is regulated directly by c-Myc DNA binding via phosphatidylinositol 3-kinase signaling, thus revealing a previously uncharacterized mechanism of Ha-ras-mediated oncogenesis through AEG-1.

tumor-promoting gene | signaling pathway | transcription

The *ras* protooncogene is a small GTP/GDP-binding protein that plays a critical role in cell growth control as a central component of mitogenic signaling (1). Ras activation initiates a complex array of signal transduction pathways including the Raf/MAPK (ERK) pathway, primarily involved in plasma-membrane-to-nucleus signaling crucial for mitogen-induced cell proliferation (2, 3); the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, which is involved in cell survival signaling (4); the Rac/Rho pathway, involved in cytoskeletal remodeling (5); and Rac/JNK and Rac/p38 pathways, both of which appear to be involved in cell stress responses, growth inhibition, and apoptotic signaling (6–8). Activation of Ras signaling pathways is essential for cells to exit a quiescent state and pass through the G_1 phase of the cell cycle (9). Under normal conditions, the action of Ras and other members of the Ras pathway are stringently regulated during the cell cycle and under different growth conditions (10). In a tumor cell, the oncogenic activation of *ras* is a consequence of point mutations that either impair GTPase activity or enhance GTP-binding affinity, resulting in a highly active proliferative signal (1). In addition, it is possible that the downstream protein targets of that signal transduction pathway might be expressed abnormally. *Ras* mutations are found in a wide variety of human cancers (11). Therefore, aberrant Ras signaling represents a nodal pathway regulating tumor-cell growth and providing a potential target for cancer therapy (12, 13).

We recently reported the cloning and functional characterization of an HIV-1-inducible gene, astrocyte elevated gene-1 (*AEG-1*), which is induced in primary human fetal astrocytes infected with HIV-1 or treated with gp120 or TNF-α (14–17). Intriguingly, AEG-1 induces increased anchorage-independent growth and invasiveness of tumor cells and increased expression of adhesion molecules by activating the NF-κB pathway, and AEG-1 can physically interact with p65 and modulate its function in the nucleus (17). AEG-1 expression also is elevated in subsets of breast carcinomas, malignant gliomas, and melanomas, and it synergizes with oncogenic Ha-ras to enhance soft-agar colony-forming ability of nontumorigenic immortalized melanocytes (16). In addition, AEG-1 expression is elevated in adult astrocytes transformed by sequential overexpression of simian virus 40 T/t antigen, telomerase (hTERT), and oncogenic Ha-ras, thereby displaying an aggressive glioma-like phenotype (16, 18). These results strongly suggest that AEG-1 might be functionally related with oncogenic Ha-ras and that it plays a critical role in Ha-ras-mediated oncogenesis.

In the present study, we examined the effects of oncogenic Ha-ras on AEG-1 expression. AEG-1 expression was markedly induced by Ha-ras, and this induction was mediated transcriptionally through the PI3K signaling pathway. Activation of two E-box elements in the *AEG-1* promoter by increased c-Myc binding was shown to be critical for this Ha-ras-mediated AEG-1 induction. We also documented that *AEG-1* siRNA inhibited Ha-ras-mediated colony formation. Although the cooperative effect of Ha-ras and c-Myc in controlling gene expression is well established, this article is a demonstration that Ha-ras-induced increased expression of a tumor-promoting gene is mediated by direct DNA binding of c-Myc upon activation of PI3K signaling. Our findings uncover a previously uncharacterized mechanism of Ha-ras-mediated tumorigenesis and delineate a crucial role of *AEG-1* in promoting cancer development and/or maintenance. In these contexts, AEG-1 may provide a viable target for therapeutic intervention in ras-mediated pathogenicity.

Results

Human *AEG-1* Is Induced by Oncogenic Ha-*ras*. To investigate whether oncogenic Ha-ras induces AEG-1 expression, the effect of overexpression of Ha-ras on AEG-1 protein level was determined. We used THV cells, which are human adult astrocytes immortalized by simian virus 40 T/t antigen and hTERT; THR cells, which are THV cells containing a stable overexpression of Ha-ras (18); cloned rat embryo fibroblasts (CREF); and CREF stably overexpressing Ha-ras (CREF-ras) (19). As shown in Fig. 1*A*, transient transfection of a Ha-ras expression plasmid but not the empty vector (pcDNA) resulted in significant induction of AEG-1 protein in THV cells. As a corollary, AEG-1 levels were found to be significantly higher in THR and CREF-ras cells

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The authors declare no conflict of interest.

Abbreviations: *AEG-1*, astrocyte elevated gene-1; PI3K, phosphatidylinositol 3-kinase; CREF, cloned rat embryo fibroblast; MEK, MAPK/ERK kinase; CREB, cAMP-response element-binding protein.

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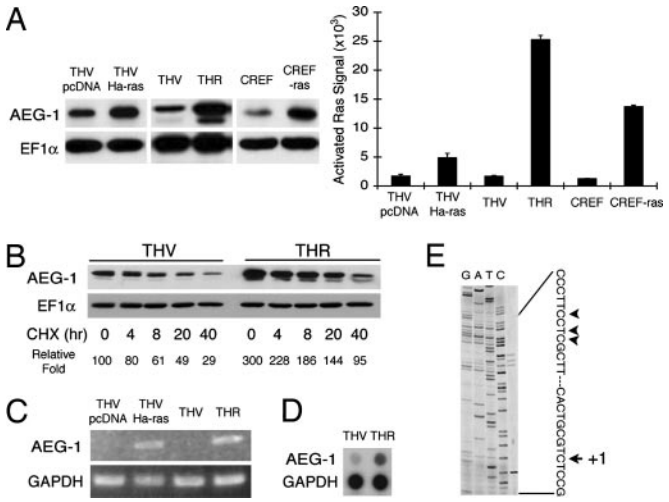

Fig. 1. Oncogenic Ha-ras induces AEG-1 expression. (*A*) THV cells were transfected with pcDNA3.1/Hygro(+) or T24 Ha-ras expression vector. Cell lysates were prepared from pcDNA3.1- or Ha-ras-transfected THV, THR, CREF, and CREF-ras cells. The expression of AEG-1 and Ras was analyzed by Western blot analysis (*Left*) and Ras activation assay (*Right*). Graph data are presented as mean ± SD. (*B*) THV and THR cells were treated with cycloheximide (CHX; 50 μg/ml) for 0 to 40 h. Cells were lysed immediately, and cell lysates were subjected to Western blot analysis. Values are presented as relative AEG-1 expression versus EF1α expression compared with control untreated THV cells taken as 100. (*C*) Total RNA was isolated from pcDNA3.1- or Ha-ras-transfected THV and THR cells, and then RT-PCR was conducted. (*D*) Nuclei were prepared from THV and THR cells. The isolated nuclei were used to label preinitiated RNA transcription with [α-^{32}P]UTP *in vitro*, and the purified RNA then was hybridized to a dot blot carrying an equivalent amount of panel DNA probes. The transcription rate of GAPDH served as control. (*E*) Five micrograms of total RNA extracted from primary human fetal astrocytes was mixed with the 5′ end-labeled oligonucleotide, and cDNA was synthesized by reverse transcriptase. The extension products were separated on a gel along with a sequencing ladder by using the same primer and the cloned human *AEG-1* 5′ upstream region in pGL3-AEG1prom as a size marker. The major transcriptional initiation site, indicated by the arrow, is taken as +1, and other transcriptional initiation sites are indicated by arrowheads.

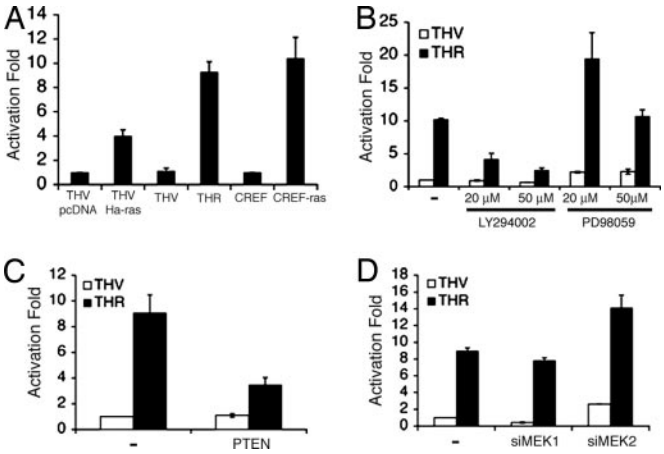

Fig. 2. Oncogenic Ha-ras activates the human *AEG-1* promoter through the PI3K signaling pathway. (*A*) THV cells were transfected with pGL3-AEG1prom and T24 Ha-ras expression vector together with pSV-β-gal as an internal control. A parental CMV vector, pcDNA3.1, was used as a negative control. THV and THR cells were transfected with the pGL3-AEG1prom together with pSV-β-gal as an internal control. Two days after transfection, cells were harvested, and extracts were prepared to measure luciferase and β-galactosidase activities. Values are presented as fold normalized activity relative to that of control vector pcDNA3.1 taken as 1. CREF and CREF-ras cells were transfected with pGL3-AEG1prom together with pSV-β-gal as an internal control. Values are presented as fold normalized activity relative to that in CREF cells taken as 1. (*B*) THV and THR cells were transfected with pGL3-AEG1prom together with pSV-β-gal as an internal control. One day after transfection, cells were treated with a PI3K inhibitor, LY294002, or a MEK inhibitor, PD98059, as indicated for 16 to 18 h before harvesting. Values are presented as fold normalized activity relative to that in the mock-treated (−) THV cells taken as 1. (*C*) THV and THR cells were transfected with pGL3-AEG1prom and PTEN expression vector together with pSV-β-gal as an internal control. Values are presented as fold normalized activity relative to that of control vector pcDNA3.1 in THV cells taken as 1. (*D*) THV and THR cells were transfected with pGL3-AEG1prom and control, *MEK1*, or *MEK2* siRNA (−, siMEK1, or siMEK2, respectively) together with pSV-β-gal as an internal control. Values are presented as fold normalized activity relative to that of control siRNA in THV cells taken as 1. Graph data are presented as mean ± SD.

when compared with THV and CREF cells, respectively, indicating that AEG-1 expression is induced by oncogenic Ha-ras. Ras activity assays demonstrated high active ras levels in THR and CREF-ras cells versus THV and CREF cells, respectively, and moderate induction of active ras upon transient transfection of Ha-ras into THV cells (Fig. 1*A*). The level of active ras correlated with the AEG-1 protein level, further confirming Ha-ras as a positive regulator of AEG-1 expression.

Given that most genes are regulated at multiple levels, including protein stability and mRNA synthesis, we determined whether Ha-ras-induction of AEG-1 protein was mediated by an increase in protein stability. THV and THR cells were treated with 50 μg/ml of cycloheximide for 0 to 40 h to block *de novo* protein synthesis. Under these conditions, the half-life of AEG-1 protein was ≈20 h in both THV and THR cells (Fig. 1*B*), indicating that AEG-1 protein is very stable, and its stability is not modulated by Ha-ras overexpression.

To determine whether AEG-1 protein accumulation mediated by Ha-ras was associated with an increase in AEG-1-specific mRNA expression, total RNA from THV-pcDNA, THV-Ha-ras, THV, and THR cells was isolated and examined by RT-PCR. As shown in Fig. 1*C*, *AEG-1* mRNA was increased significantly in THV-Ha-ras and THR cells when compared with THV-pcDNA and THV cells, respectively. This induction in AEG-1 mRNA expression was caused by enhanced transcription as confirmed by performing nuclear run-on assays (Fig. 1*D*). These results show that human AEG-1 is induced by oncogenic Ha-*ras* at a transcriptional level.

To determine the transcriptional initiation sites of *AEG-1* and construct an *AEG-1* promoter-reporter plasmid, we isolated the 5′ upstream region of the *AEG-1*, which is located at chromosome 8q22.1, where cytogenetic analysis of human gliomas indicated recurrent amplification (see *Supporting Text* and Fig. 6, which are published as supporting information on the PNAS web site). As shown in Fig. 1*E*, we identified four transcription initiation sites by using primer extension analysis. The intensity of the proximal band was stronger than that of the other three bands. As such, we determined the cytosine residue of the proximal band as the transcription start site (+1). Thus, the cloned fragment from genomic PCR includes −2,710 to +49 in the 5′ upstream region of the *AEG-1* gene.

Oncogenic Ha-ras Activates the Human *AEG-1* Promoter by the PI3K Pathway. To investigate the role of Ha-ras in activation of the human *AEG-1* promoter, pGL3-AEG1prom was transiently transfected into THV cells with a T24 Ha-ras-expression plasmid. Ha-ras overexpression resulted in a ≈4-fold increase in human *AEG-1* promoter activity when compared with transfection of the control plasmid (pcDNA) (Fig. 2*A*). Similarly, *AEG-1* promoter activity was ≈8- to 10-fold higher in THR and CREF-*ras* cells than in THV and CREF cells, respectively, thus demonstrating that the *AEG-1* promoter has a significant transcriptional response to the activated Ha-ras pathway (Fig. 2*A*).

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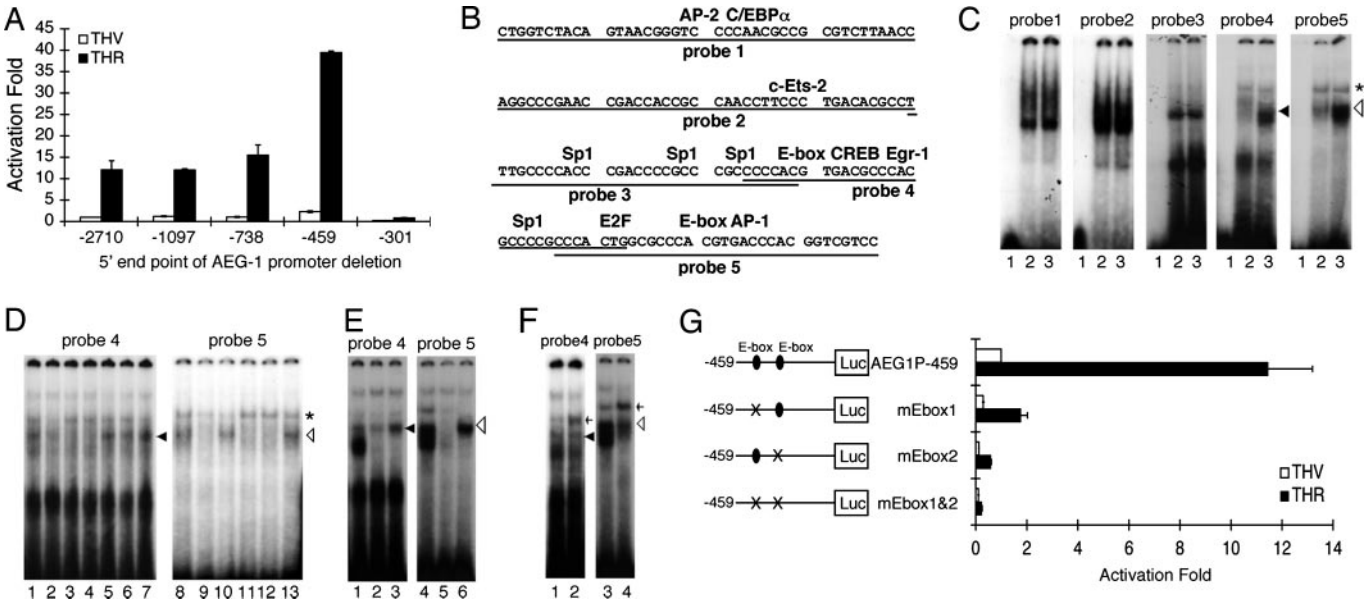

Fig. 3. Two E-box elements are necessary for oncogenic Ha-ras-mediated *AEG-1* promoter activation. (*A*) THV and THR cells were transfected with different *AEG-1* promoter deletion-reporter constructs together with pSV-β-gal as an internal control. Values are presented as fold normalized activity relative to that of full-length promoter (−2,710) in THV cells taken as 1. (*B*) Sequences of the −459/−302 region in the human *AEG-1* promoter are presented. The positions of five oligonucleotide probes used in EMSA are underlined, and putative transcription factor binding sites are indicated. (*C*) THV and THR nuclear extracts were mixed with each radiolabeled oligonucleotide for EMSA as indicated. Lane 1, no extract added; lane 2, THV nuclear extracts; lane 3, THR nuclear extracts. (*D*) For competition assays, a 25-fold excess of corresponding unlabeled probe (lanes 2 and 9) or 100 nM of each consensus competitor (lanes 3 and 11, c-Myc/Max; lanes 4 and 12, USF-1; lane 5, CREB; lane 6, Egr-1; lane 7, Sp1; lane 10, E2F; and lane 13, AP-1) was added. Lanes 1 and 8, no competitor added. THR nuclear extracts and probe 4 (lanes 1–7) or probe 5 (lanes 8–13) were used as indicated. (*E*) A 25-fold excess of unlabeled probe encompassing mutated E-box [lanes 1 and 4, no competitor; lane 2, unlabeled probe 4; lane 3, P4M (probe 4 containing mutated E-box); lane 5, unlabeled probe 5; and lane 6, P5M (probe 5 containing mutated E-box)] was added for competition assays. THR nuclear extracts and probe 4 (lanes 1–3) or probe 5 (lanes 4–6) were used as indicated. (*F*) Supershift analysis was performed with c-Myc antibody (lanes 1 and 3, no antibody added; and lanes 2 and 4, c-Myc antibody). THR nuclear extracts and probe 4 (lanes 1 and 2) or probe 5 (lanes 3 and 4) were used as indicated. The arrow indicates a supershifted band by anti-c-Myc antibody. (*G*) THV and THR cells were transfected with each mutant reporter together with pSV-β-gal as an internal control. Values are presented as fold normalized activity relative to that of AEG1Prom-459 in THV cells taken as 1. Crosses indicate site-specific mutations in two E-box elements. Graph data are presented as mean ± SD.

To determine the intracellular signaling pathway by which Ha-ras induces *AEG-1* promoter activity, a PI3K inhibitor, LY294002, and a MAPK/ERK kinase (MEK) inhibitor, PD98059, were used. The addition of LY294002, but not PD98059, significantly attenuated Ha-ras-mediated *AEG-1* promoter activation in THR cells with little change in basal *AEG-1* promoter activity in THV cells (Fig. 2*B*). PTEN is a phosphatase antagonizing the diverse downstream signaling effector pathways activated by PI3K-derived phospholipids (20, 21). Cotransfection of a PTEN expression plasmid with pGL3-AEG1prom also significantly attenuated Ha-ras-induced *AEG-1* promoter activity in THR cells without affecting the basal promoter activity in THV cells (Fig. 2*C*). As observed with PD98059, siRNA for MEK1 and MEK2 showed no inhibitory effect on Ha-ras-induced *AEG-1* promoter activity (Fig. 2*D*). These results indicate that the PI3K signaling pathway is involved in Ha-ras-mediated *AEG-1* promoter activation. Of note, inhibition of the MEK pathway slightly increased Ha-ras-mediated *AEG-1* promoter activation, the significance of which remains to be determined.

Identification of cis Elements in the Human *AEG-1* Promoter Required for Response to Ha-ras. The results described above indicate that the human *AEG-1* promoter is activated by Ha-ras, and this activation is mediated by the PI3K signaling pathway. We next determined in more detail the cis elements in the *AEG-1* promoter essential for response to Ha-ras. A series of 5′ deletion mutants of the *AEG-1* promoter-reporter construct were transiently transfected into THV and THR cells, and promoter activity of each deletion-reporter construct was examined (Fig. 3*A*). Serial deletions from −2,710 to −459 showed a ≈3-fold increase in promoter activity in both cell lines, indicating the presence of negative transcriptional control elements in this region (−2,710/−459). Deletion from −459 to −301 resulted in a >90% loss of basal as well as Ha-ras-induced promoter activity in THV and THR cells, respectively. These results suggest that transcription factors binding to this region (−459/−301) are capable of regulating basal *AEG-1* promoter activity as well as its induction in response to Ha-ras.

We next identified the transcription factors binding to the −459/−301 region by using electrophoretic mobility shift assays (EMSA). Five double-stranded oligonucleotide probes, each 30- to 40-bp in length and containing putative transcription factor binding sites, were generated to span the region from −459 to −302 of the *AEG-1* promoter (Fig. 3*B*). As shown in Fig. 3*C*, all of the probes generated at least one DNA–protein complex from THV and THR nuclear extracts. The intensity of DNA–protein complexes generated by probes 1, 2, and 3 did not show any significant change with THV and THR nuclear extracts. However, the intensity of one band generated by probes 4 and 5 was significantly higher with THR nuclear extracts as compared with THV nuclear extracts (denoted as complexes I and II with filled and open arrowheads in Fig. 3*C*, respectively). With probe 5, an additional band was identified (complex III, indicated by the asterisk in Fig. 3*C*) that did not show any differential binding pattern between THV and THR cells. These results indicate that the region −356/−302 of the *AEG-1* promoter binds to Ha-ras-activated transcription factors. This region contains several putative transcription factor binding sites, including two E-box elements to which the basic helix–loop–helix (bHLH) proteins such as c-Myc and USF-1 bind, as shown in Fig. 3*B*.

To further characterize the increased DNA–protein complexes I and II from THR nuclear extracts, unlabeled probe 4, probe 5, and several consensus oligonucleotides for Myc/Max, USF-1, cAMP-response element-binding protein (CREB), Egr-1, Sp1, E2F, and AP-1 were used as competitors in EMSA (Fig. 3*D*). Shifted bands that were not competed out after addition of unlabeled probes 4 and 5 were considered as nonspecific binding. The intensity of complex III was reduced significantly by an E2F consensus oligonucleotide, indicating that the human *AEG-1* promoter might have an E2F transcription factor binding site (Fig. 3*D*, lane 10). Interestingly, consensus oligonucleotides for the transcription factors Myc/Max and USF-1 completely competed both complexes I and II (Fig. 3*D*, lanes 3, 4, 11, and 12). In contrast, the other consensus oligonucleotides had no effect on nucleoprotein complex formation. In addition, complexes I and II were not affected by unlabeled probe 4 and probe 5, each containing a mutated E-box element (Fig. 3*E*, lanes 3 and 6). These results indicate that E-box elements confer Ha-ras-mediated differential transcription factor binding. Supershift experiments with anti-Myc antibody resulted in retarded mobility of complexes I and II (indicated by arrows in Fig. 3*F*), demonstrating the presence of c-Myc in these complexes (Fig. 3*F*, lanes 2 and 4). All these data indicate that Ha-ras increases binding of Myc/Max to the E-box elements in the *AEG-1* promoter.

To clarify the roles of the two E-box elements in the −356/−302 region of the *AEG-1* promoter, three mutant *AEG-1* promoter-reporter constructs containing mutations in the E-box elements were engineered (Fig. 3*G*). These constructs were transiently transfected into THV and THR cells, and promoter activities were compared with that of the wild-type plasmid AEG1prom-459. As shown in Fig. 3*G*, mEbox1 and mEbox2 mutants still responded to Ha-*ras*, although the extent of activation was reduced by >60%. Each mutation also significantly reduced the basal *AEG-1* promoter activity. In contrast, the mEbox1&2 mutant containing mutations in both E-box elements abolished response to Ha-*ras*. These results indicate that both E-box elements are critical for basal *AEG-1* promoter activity as well as Ha-ras-mediated *AEG-1* promoter activation.

PI3K/Myc Signaling Pathway Mediates Ha-ras-Induced AEG-1 Expression. AKT has been identified as a physiologically relevant kinase acting downstream of PI3K to phosphorylate and inactivate GSK3β, which negatively controls Myc activity (21–23). To confirm that PI3K signaling is involved in oncogenic Ha-ras-induced AEG-1 expression, we examined phosphorylation of AKT and GSK3β by Western blotting analysis using THV and THR cell lysates treated with LY294002. Despite a similar level of AKT and GSK3β expression, THR cells exhibited a high-level phosphorylation of AKT and GSK3β when compared with THV cells, and this phosphorylation was inhibited by LY294002 treatment (Fig. 4*A*). Accordingly, Myc, but not USF-1, protein level was higher in THR cells than in THV cells, and LY294002 reduced Myc protein levels in THR cells, demonstrating a correlation between GSK3β phosphorylation by Ha-*ras*-mediated AKT activation and Myc expression in THR cells (Fig. 4*A*). Reduction of *AEG-1* expression by LY294002 was confirmed at the protein level (Fig. 4*A*) as well as at the transcription level (Fig. 2*B*).

To ascertain a functional role of c-Myc in AEG-1 expression, 50 nM of c-*myc* siRNA was transfected into THV and THR cells. As shown in Fig. 4*B Upper*, siRNA targeting c-*myc* completely inhibited expression of c-Myc protein in THV and THR cells and also significantly decreased AEG-1 protein expression. In addition, siRNA of c-*myc* completely inhibited induction of *AEG-1* promoter activity by Ha-ras and also significantly decreased the basal *AEG-1* promoter activity in both THV and THR cells (Fig. 4*B Lower*). Furthermore, overexpression of Ha-ras or c-Myc alone augmented *AEG-1* promoter activity, which was synergistically induced when c-Myc and Ha-ras expression plasmids were cotransfected into target cells (Fig. 4*C*). These results indicate that c-Myc plays an important role in Ha-ras-mediated AEG-1 induction as well as its basal promoter activity.

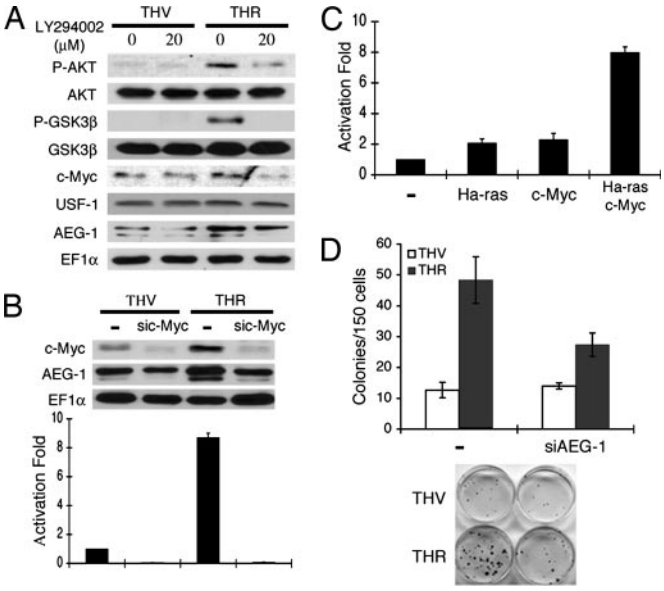

Fig. 4. Transcription factor c-Myc is the mediator of oncogenic Ha-ras-mediated AEG-1 induction. (*A*) Cell lysates from THV and THR cells treated with 20 μM of LY294002 for 2 days were immunoblotted with the indicated antibodies. (*B*) THV and THR cell lysates transfected with control or c-*myc* siRNA (− or sic-Myc) were immunoblotted with c-Myc, AEG-1, and EF1α antibodies (*Upper*). THV and THR cells were cotransfected with pGL3-AEG1prom and control or c-*myc* siRNA as indicated together with pSV-β-gal as an internal control. Values are presented as fold normalized activity relative to that of control taken as 1 (*Lower*). (*C*) THV cells were cotransfected with pGL3-AEG1prom, Ha-ras, and c-Myc expression vector as indicated together with pSV-β-gal as an internal control. Values are presented as fold normalized activity relative to that of control vector pcDNA3.1 (−) taken as 1. Graph data are presented as mean ± SD. (*D*) AEG-1 is required for Ha-ras-mediated colony formation. THV and THR cells were transfected with control or *AEG-1* siRNA. The data are represented as mean ± SD and represent three independent experiments in triplicate.

AEG-1 Is Required for Ha-ras-Mediated Colony Formation. Previous studies indicate that AEG-1 has a proliferative modulating effect in immortalized melanocytes and HeLa cells (16, 17). The present results indicate that AEG-1 is a target of oncogenic Ha-ras signaling through the PI3K pathway. To determine whether AEG-1 may mediate the proliferative effect of oncogenic Ha-ras, we inhibited AEG-1 expression in THV and THR cells by using siRNA and examined the effect of such treatment on colony formation. *AEG-1* siRNA significantly reduced AEG-1 expression (Fig. 7, which is published as supporting information on the PNAS web site). As shown in Fig. 4*D*, the number and size of colonies in THR cells were greater and bigger than in THV cells, indicating an effect of oncogenic Ha-ras on cell proliferation. Transfection with *AEG-1* siRNA resulted in a ≈40% reduction in colony formation in comparison with transfection with control siRNA in THR cells, without affecting THV cells (Fig. 4*D*). These results indicate that AEG-1 plays an essential role as a downstream target gene in oncogenic Ha-*ras*-mediated proliferation and transforming activities.

Discussion

Previous studies revealed that AEG-1 might be important for tumor development, progression, and metastasis through acti-

vation of the NF-κB pathway and its cooperation with oncogenic Ha-ras signaling pathways (16, 17). In the present study, we demonstrate that AEG-1 is a downstream target gene of Ha-ras. We show that Ha-ras-mediated AEG-1 induction is regulated mainly at the transcriptional level rather than by modulating protein stability. This induction was attenuated by treatment with LY294002 or PTEN overexpression, indicating that activation of the PI3K signaling pathway regulates Ha-ras-mediated AEG-1 induction. We also observed that *AEG-1* expression was mildly elevated by treatment with the MEK inhibitor PD98059 and siRNA of MEK2 (Fig. 3 *B* and *D*). Recent studies demonstrate that blockade of ERK1/2 with PD98059 causes induction of specific genes, such as IL-6, CYP1A1, and NF-κB, even though other downstream Ras signaling pathways induce their activity (24–26). Cellular response to any stimulus may be affected by cell type, developmental stage, and extracellular conditions. The mechanisms underlying different cellular responses are only partially understood and can be explained in certain cases by differential gene and protein expression patterns. The complex relationships among diverse extracellular conditions and cellular responses to a distinct stimulus have only recently begun to be elucidated at the mechanistic level. It will be very interesting to delineate the potential cross-talk relationship between the PI3K and MEK signaling pathways in regulating Ha-ras-mediated AEG-1 expression.

From the promoter analysis of *AEG-1*, c-Myc binding to two E-box elements is critical for Ha-ras-mediated *AEG-1* promoter activation as well as for basal promoter activity. A number of genes involved in tumor progression and metastasis, such as osteopontin, cdc2, connexin 43, and MMP-9, have been shown to be augmented by the cooperative action of Ras and c-Myc (27–31). However, as yet no study has demonstrated that the induction of a ras-responsive gene is mediated by direct binding of c-Myc to the gene promoter. In these contexts, our studies add a dimension to the molecular circuitry in the *ras*–c-*myc* axis. We also confirmed that the human *AEG-1* promoter has positive (−459/−302) and negative (−738/−460) regulatory regions. Although the positive regulatory region contains several putative transcription factor binding sites critical for basal promoter activity, such as Sp1, E-box element, CREB, and Ets-2 (32–35), our data indicate that two E-box elements in this region are functional and important for both basal and Ras-induced promoter activity. The negative regulatory region has putative RAR-α and YY1 binding sites that have been shown to act predominantly as repressors of transcription (36–39). Further studies will be needed to elucidate the involvement of these transcription factors in mediating transcriptional repression of *AEG-1*.

We demonstrate that Ha-ras mediates AEG-1 induction through the PI3K/GSK3β/c-Myc signaling pathway. PI3K/AKT signals control several growth-regulatory transcription factors. Two prominent examples are the forkhead box (FoxO) protein and NF-κB. Other transcriptional regulators whose activities are affected by PI3K/AKT signaling include MIZ-1, p53, AP-1, c-Myc, β-catenin, and HIF1α (21). The exact roles of these proteins during PI3K-mediated oncogenesis currently are unknown, but they have all been linked to oncogenic transformation. AKT phosphorylates and thereby inactivates the cell-cycle inhibitor MIZ1 and also suppresses p53 activity by a mechanism that involves MDM2. By contrast, the activity of AP-1, c-Myc, and β-catenin are increased by AKT. These targets are negatively controlled by GSK3β, which is inactivated by AKT-mediated phosphorylation (21). The present study confirms that activated PI3K signaling in THR cells phosphorylates AKT and GSK3β, which activates c-Myc, not USF-1, resulting in increased AEG-1 expression (Fig. 5).

Human *AEG-1* also has been cloned as metadherin, and cloning of the mouse (3D3) and rat (lyric) homologues of *AEG-1* has been reported (40–42). Rat lyric was reported as an overexpressed gene in rat liver and colon tumors (40, 41). Human metadherin manifests high expression in breast cancer cells and contains a lung-homing domain that facilitates metastasis of breast cancer cells to the lungs (42). Our previous experiments indicated that AEG-1 increased anchorage-independent growth and invasiveness of tumor cells and increased expression of adhesion molecules by activating the NF-κB pathway (17). We also confirmed elevated levels of AEG-1 in subsets of breast cancer, glioblastoma multiforme, and melanoma cells, and demonstrated that ectopic overexpression of AEG-1 promoted anchorage-independent colony-forming ability of immortalized melanoma cells synergistically with oncogenic Ha-ras (16). A recent report demonstrated that Ras cooperates with *Myc* in tumorigenesis in relation to hTERT, p53, retinoblastoma (RB), and PTEN (43). In addition, we now document that AEG-1 expression is significantly induced by oncogenic Ha-ras through the PI3K/AKT/GSK3β/c-Myc signaling pathway (Fig. 5), and *AEG-1* siRNA inhibits the colony-formation activity of oncogenic Ha-ras. Together, we suggest that AEG-1 is one of the downstream target genes of the oncogenic Ha-ras signaling pathway and may play an important role in Ha-ras-mediated carcinogenesis. Increased insights into the detailed molecular mechanism of AEG-1 function and regulation will help clarify its role in the process of tumorigenesis and facilitate development of therapeutic strategies by targeting AEG-1 via antisense, siRNA, or a small-molecule inhibitor for inactivation or by using the *AEG-1* promoter linked to tumor-suppressor genes, such as p53 and *mda*-7/IL-24 (44, 45) for gene therapy of cancers having activated Ras.

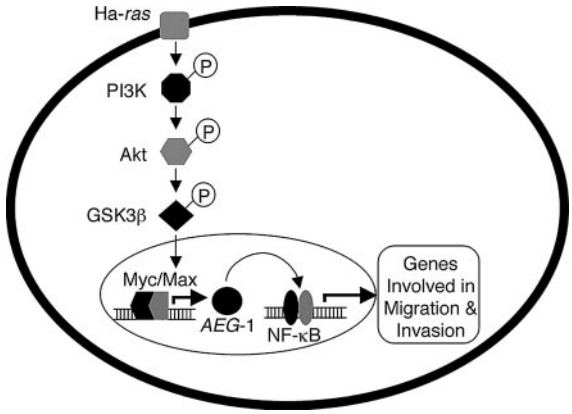

Fig. 5. Hypothetical model of signal transduction pathways involved in Ha-ras-mediated AEG-1 induction. Ha-ras activates the PI3K signaling cascade, resulting in increased binding of Myc/Max to the *AEG-1* promoter, and augments AEG-1 expression. AEG-1 activates the NF-κB pathway that regulates expression of genes involved in migration and invasion and thus plays a crucial role in Ha-ras-mediated tumor progression.

Materials and Methods

Cell Cultures and Reagents. Primary human fetal astrocytes were isolated and cultured as described in ref. 14. CREF and CREF-ras cell lines were previously described (19). THV and THR cells were kind gifts of J. N. Rich and C. M. Counter (Duke University Medical Center, Durham, NC) (18). Cycloheximide, LY294002, and PD98059 were purchased from Calbiochem (San Diego, CA).

Plasmids and siRNA. The constructions of *AEG-1* promoter-reporter plasmids and the PTEN expression vector are described in *Supporting Methods* in *Supporting Text*. The T24 Ha-ras and c-Myc expression vectors were described previously (16, 46). The control and *c-myc* siRNA were purchased from Ambion (Austin,

TX). The *MEK1* siRNA, *MEK2* siRNA, and *AEG-1* siRNA were generated by using the Silencer siRNA Construction kit (Ambion, Austin, TX) according to the manufacturer's instructions. The sequences of primers used are described in Table 1, which is published as supporting information on the PNAS web site.

Western Blotting Analysis and Ras Activity Assay. Whole-cell lysates were prepared, and Western blotting analysis was performed as previously described (17). Ras activities were measured colorimetrically by using the Ras GTPase Chemi ELISA kit (Active Motif, Carlsbad CA) according to the manufacturer's instructions.

RT-PCR, Nuclear Run-On Assay, and Primer Extension Assay. Total RNA was extracted from cells by using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Two micrograms of total RNA was used for RT-PCR following standard methods. Nuclear run-on assays and primer extension assays were performed as described in ref. 47. The primers used are described in Table 1.

Transient Transfection and Luciferase Assays. A total of 1×10^5 cells were seeded per well in 24-well plates and transfected with 800 ng of total DNA by using LipofectAMINE 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Luciferase assays were performed as described in ref. 17. Luciferase activity was normalized by β-galactosidase activity, and the data presented are the fold activation $\pm$ SD from at least three independent experiments performed in duplicate or triplicate.

EMSA. Nuclear extracts were fractionated by the modified Schreiber's method, and EMSA was performed as previously described (17). The oligonucleotides used as probes are indicated in Fig. 3*B*. For competition experiments, unlabeled probes or several consensus oligonucleotides for Myc/Max, USF-1, CREB, Egr-1, Sp1, E2F, and AP-1 (Santa Cruz Biotechnology, Santa Cruz, CA) were added 10 min before addition of the labeled probes. The double-stranded oligonucleotides containing a mutated E-box element (P4M and P5M; see Table 1) also were used for competition. For supershift experiments, 2 μl of anti-c-Myc antibody (sc-764X; Santa Cruz Biotechnology, Santa Cruz, CA) was added to the reaction mixture and was incubated for 30 min at 4°C before the addition of the probe.

Colony-Formation Assay. THV and THR cells were plated at a density of 1×10^6 cells per 6-cm dish, and 1 day later were transfected with 50 nM of control or *AEG-1* siRNA. After 2 days, the cells were trypsinized and counted, and 150 cells were plated in 6-cm dishes. Colonies of >50 cells were scored after 10 days.

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